Purging of Contaminating Breast Cancer Cells from Hematopoietic Stem Cell Grafts by Adenoviral GAL-TEK Gene Therapy and Magnetic Antibody Cell Separation

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ABSTRACT
The presence of contaminating tumor cells in autologous bone marrow or peripheral blood stem cell (PB-SC) preparations increase the likelihood of relapse in women receiving transplants for metastatic breast cancer. We describe a new technique for purging breast cancer cells (BCCs) that combines two independent strategies: (a) the specific enrichment of CD34+ progenitor stem cells by magnetic antibody cell separation (MACS), and then (b) infection of the contaminating BCCs with a recombinant adGAL-TEK marker/suicide gene adenovirus (ad-v), followed by the addition of ganciclovir (GCV). Infection with this ad-v results in three to four times greater expression of ad-v-delivered reporter gene in BCCs than in CD34+ cells. In addition, -2 h, -low multiplicity of infection (50:1) adGAL-TEK infections of BCC lines (MCF-7 and BT474) eradicated >99% of BCCs after 72 h of exposure to 20 \( \mu \text{M} \) GCV. However, exposure to both adenovirus and GCV at the MOIs and doses used had little effect on hematopoietic stem cells to form colonies in colony-forming unit assays. adGAL-TEK infection in our model system (10^6–10^7 BCCs added into 10^7 HSCs) also resulted in the 3 to 5 log eradication of clonogenic BCCs after the addition of GCV. MACS enrichment/purification of CD34+ cells from PB-SC contaminated with 2 \( \times 10^6 \) to 5 \( \times 10^6 \) BCCs followed by adGAL-TEK infection and GCV addition resulted in 5–7-log depletion of clonogenic BCCs as well as enrichment of CD34+ progenitor cells to >98%, with the recovery of >70% of hematopoietic stem cells. This adenoviral purging system is so robust that poor MACS purification, resulting in 1.5-log depletion of BCCs, still permits excellent ad-v infection and BCC killing.

INTRODUCTION
High-dose chemotherapy, followed by the autologous transplantation of BM or PB-SCs is a treatment option for patients with breast cancer (1–4). Although this approach results in prolonged survival of a subgroup of such patients, most eventually relapse. One potential source of relapse is the reinvasion of tumor cells that can contaminate the HSC preparations (1, 2). Various methods have been devised to eliminate tumor cells from BM transplant or PB-SC preparations (3, 4). Present methods include mechanical, chemical, and immunological means (5–7). However, although these methods may significantly reduce tumor cell contamination of BM (2–5 logs of BCCs), they are generally complex, labor intensive, and generally toxic to hematopoietic progenitor cells. Most importantly, the utility of these methods is contingent on the high-level expression of cancer cell surface markers (8, 9), and tumor heterogeneity may result in major batch-to-batch variability.

On a different note, by delivering therapeutic genes to cancers, ad-v may be useful in the therapy of cancer (10–12). These vectors efficiently target a wide variety of cell lines (13, 14) and animal tissues (10, 15, 16). As part of this effort, recombinant adenoviruses have been created to deliver a wide variety of therapeutic genes. One strategy is to deliver “suicide genes” that encode an enzyme such as the HSVtk (17) that can activate the nontoxic prodrg GCV (18, 19) within tumor cells and render the tumor cell sensitive.

Of relevance from the standpoint of purging methods, recent data from studies conducted by Wattel (20) and Seth et al. (21) suggest that HSCs and high-density BM cells lack the capacity for efficient ad-v infection. This raises the possibility that in cocultures of target tissues or cells and HSCs, ad-v will preferentially infect the target tissue or cells (i.e., epithelium) but spare the HSCs; we therefore developed a purging technique.

Received 8/19/98; revised 1/26/99; accepted 3/4/99.

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1 This work is supported through a grant from the Susan G. Komen Breast Cancer Foundation, by the M. D. Anderson Breast Cancer Research Program (to F. C. M.), and “Polo-on-the-Prairie.” Q. Y. is a WHO fellow. Additional support was provided by Grant CS-16672 from the NIH and a grant from the Stringer Professorship for Cancer Treatment and Research (to M. A.).

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3 The abbreviations used are: BM, bone marrow; PB-SC, peripheral blood stem cell; PB-MNC, peripheral blood mononuclear cell; ad-v, adenoviral vector; BCC, breast cancer cell; HSC, hematopoietic stem cell; HSVtk, herpes simplex thymidine kinase; \( \beta \)-gal, \( \beta \)-galactosidase; GAL-TEK, an in-frame fusion protein consisting of both E. coli LacZ (\( \beta \)-galactosidase) and HSVtk; GCV, ganciclovir; MACS, magnetic antibody cell separation; MOI, multiplicity of infection; CSF, colony-stimulating factor; FACS, fluorescence-activated cell sorting; RT-PCR, reverse transcription-PCR; CFU, colony-forming unit; RU, relative unit; RLU, relative light unit; RT-PCR, reverse transcription-PCR.
that exploits this phenomenon. Since our initial description of this purging concept in an abstract (22), two additional reports on the utility of ad-v for purging contaminating epithelial cells have been published (23, 24). However, neither of these reports suggest useful clinical applications for this purging technology, because ad-v purging of unconcentrated PB-SC is superfluous, because many of the cells in the PB-SC collection do not contribute to engraftment, and these unselected cell applications would require approximately 2 logs more ad-v/ml of PB-SC for these purging techniques to be successful.

There are new techniques that can enrich the population of HSCs in BM or PB-SC preparations that can also be used to positively select CD34+ cells or immunomagnetically deplete contaminating cells. The MACS technique is one such technique. It has been used to immunomagnetically select CD34+ cell populations to a greater than 95% purity (25) while reducing background CD34 nonexpressing cells (or contaminating BCCs) by 3–5 logs (26).

Recognizing that these two techniques could be combined, we created a novel BCC purging technique that involves first the specific enrichment of stem cell progenitor CD34+ cells in the autograft by MACS purification, followed by infection of the CD34+–enriched population by a recombinant ad-v carrying a suicide gene, which preferentially infects contaminating epithelial cells but not HSCs. These ad-v–infected cells are then forced into cell suicide upon the addition of GCV. These combined techniques can reduce the number of contaminating BCCs by 5–7 logs while enriching the HSCs to >98% purity. We present here a useful clinical application that can be easily incorporated into many BM or pheresis protocols. This combined enrichment/purge is quick (start-to-finish time, <5 h), easy, and requires currently available reagents. This novel purging technique should improve the clinical results of PB-SC transplantation in patients with breast cancer.

### MATERIALS AND METHODS

#### Cell Culture

The BCC lines, MCF-7 and BT474, were obtained from American Tissue Type Culture Collection (Rockville MD) and grown as monolayers in recommended culture medium supplemented with 10% FCS, 2 mm glutamine, and 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells for all experiments were harvested in log phase growth.

#### Fresh Patient Samples

Patient samples were obtained from the pathology laboratory after surgical resection. The metastatic tumor chunks were first diced into 2 mm3 pieces and digested for 30 min in 0.25% trypsin, 2% collagenase, and 0.25 mm EDTA. Every 10 min, the solution was removed and centrifuged, and the cells were collected. Primary cells were grown in culture medium consisting of RPMI 1640 with 15% FCS supplemented with 1× insulin-transferrin-selenium-X (Life Technologies, Inc., Grand Island, NY). Human CD34+ progenitor cell populations were acquired from either normal BM sources or PB-SC harvests. These cultures were kept viable in Myelocult H500 “complete” medium (Stem Cell Technologies, Vancouver, British Columbia, Canada). This medium is composed of α-MEM supplemented with 12.5% FCS, 12.5% horse serum, 0.2 m i-Insositol, 20 mm folic acid, 10−4 m 2-mercaptoethanol, and 2 mm glutamine.

#### Adenoviral Vectors and Infections

Recombinant ad-GAL-TEK was prepared and purified, and the titers were determined as described previously (16). Briefly, the GAL-TEK expression cassette, consisting of the cytomegalovirus I/E promoter/enhancer elements, was cloned upstream of the GAL-TEK sequence. This expression cassette was then cloned into the KpnI and NotI sites of pXCI.L1 transfer plasmid. After transfection of this plasmid with pJM17, a circular genome of E1-defective adenovirus into 293 cells, the E1-defective ad-GAL-TEK was rescued. Av1LacZ4 and Av1Lu are recombinant adenoviruses in which the reporter genes for Escherichia coli LacZ and firefly luciferase, respectively, are under the control of the Rous sarcoma virus promoter. These recombinant ad-v were generously provided by Genetics Therapy, Inc. (A Novartis Co., Gaithersburg, MD). Recombinant adenovirus was grown in AD-293 cells and purified by double cesium gradient purification.

#### Titers of Purified Adenovirus

Titers of purified adenovirus were determined by spectrophotometry and plaque assays. The high-titer ad-v was stored at −70°C in 10% glycerol, 10 mm Tris-Cl (pH 7.4), with 1 mm MgCl2. For adenoviral infections, BCCs (1 × 106 to 1 × 106 cells) and HSCs were suspended in 100 μl of PBS with 2% FCS or Terry Fox media-HCC-5150 (Stem Cell Technologies) and exposed to various MOIs of adenovirus ranging from 1:1 to 500:1 for 2 h at 37°C. After exposure, the cells were washed once in PBS with 2% FCS or Terry Fox medium-HCC-5150 and then resuspended in fresh medium. Cells were assayed for expression of the transgene 48 h after infection.

#### Cell Viability Assay

For cell viability assays, BCCs were infected in 100 μl of PBS with 2% FCS for 2 h using either adGAL-TEK or Av1LacZ4. Cells were then plated at a density of 2 × 103 cells/well in 96-well plates (Falcon, Cambridge, MA). Forty-eight h after infection, cells were exposed to varying concentrations of GCV for 72 h. Cell viability was measured using either the CELL TITER96AQ Nonradioactive Cell Proliferation assay (Promega Corp. Madison, WI) or by overlaying the cells with a solution containing 0.25% trypsin blue, examining them under 100 power and counting the number of viable cells among 100 cells/field.

#### Human HSC and BM Preparations

In most cases, HSCs were harvested from human peripheral blood after 5 days exposure to granulocyte CSF (5–10 μg/kg) to induce mobilization. These PB-SC preparations were stored in pheresis bags and delivered directly to the laboratory for use. The human BM used in our study was residual material from normal marrow was obtained from the clinic. Marrow cells were separated by Ficoll-Hypaque centrifugation at 900 × g for 15 min. Mononuclear cells were then harvested from the interface, washed twice in PBS, and cultured in Methocult-HC-5546 (Stem Cell Technologies). CD34+ cells were irradiated with 40 Gy cesium irradiation prior to use in clonogenic purging assays.

#### Reporter Gene Assays, FACS Analysis, and RT-PCR

BCCs were plated at 70% confluence, and HSCs were transduced with adGAL-TEK (β-gal and HSV-4), Av1LacZ4 (β-gal alone), or Av1Lu (firefly luciferase). Forty-eight h after infection, activity was determined in cell lysates for luciferase expression using the Luciferase assay kit (Promega Corp.) according to the manufacturer’s recommendations. β-gal activity was measured using the a β-gal assay kit (Promega Corp.) following the manufacturer’s recommendations. For FACS, adenovirus-
mediated β-gal activity was also determined by flow cytometry using the cells reacted with C_{12}-FDG (Molecular Probes, Eugene, OR). Briefly, ad-v-infected cells were equilibrated in prewarmed PBS staining medium containing 3.3 mM C_{12}-FDG with 10 mM HEPES (pH 7.5) and 4% FCS. The cells were incubated at 37°C for 30 min and then washed once in fresh, cold staining medium containing 15 mM phenylethyl D-glucopyranoside (a competitive inhibitor of β-gal), which terminates the reaction. The cells were then analyzed in a FACScan using a FITC filter sets. For RT-PCR, we performed RT-PCR analysis on MACS purified CD34+ cells and the BCC MCF-7 to detect adenovirus-specific transcripts. For this, we used the basic procedure described by Rosenfeld et al. (27), but with modifications. At 48 h after infection, cells were washed three times in PBS and solubilized in Rnasezol (Biotex Corp., Houston, TX) according to the manufacturer’s instructions. The resulting RNA preparations were digested with RNase-free DNase and subjected to RT-PCR using β-gal primers (β-gal 1 and β-gal 3) incorporating [32P]dCTP as described previously (16). For the control gene, we used the β2-macroglobulin primer set (h2-M1 and h2-M2). A 1.6% agarose gel was run to identify the resulting amplimers. Autoradiograms were developed 24 h after the gel was completed.

**Methylcellulose (Tumor Cell Clonogenic) Assay.** All clonogenic assays were performed in triplicate. The BCC lines were either treated alone or mixed with irradiated HSCs. In either case, a total of 5 × 10^5 cells were mixed into 5 ml of Methocult HCC-3430 base methylcellulose media (Stem Cell Technologies) containing α-MEM, 12% FCS, and 12% horse serum, after which the cells were plated. Where indicated, GCV was added to samples just before plating. Cultures were incubated at 37°C in 5% CO_{2}. Control BCCs in a linear dilution (5 × 10^1 to 5 × 10^5) were also plated to determine cloning efficiency.

**Hematopoietic Cell Colony Assay.** All CFU assays were performed in triplicate. Total CFUs were assayed in a methylcellulose culture system using prewarmed Methocult GF M4434 “complete” media (Stem Cell Technologies). This medium contains 50 ng/ml recombinant human stem cell factor, 10 ng/ml GM-CSF, 10 ng/ml IL-3, and 3 units/ml erythropoietin. Briefly, 5 × 10^3 treated or control HSCs/dish were plated on grided 35-mm plates (Costar) and incubated at 37°C with high humidity. The number of colonies was counted after 14 days.

**MACS.** CD34+ progenitor cell enrichment and immunomagnetic purging were performed using the MACS (Miltenyi Biotec, Auburn, CA), following the original protocol with minor modifications. Briefly, PB-SCs either alone or added with BCCs, the latter model for contaminated marrow, was reacted with Miltenyi anti-CD34 immunomagnetic colloid in filter-sterilized MACS buffer [HBSS: Ca^{2+}, Mg^{2+}, 0.5% BSA, 0.6% acid citrate dextrose-A, 100 units/ml heparin, 100–200 units/DNase (added directly before use)] for 30 min at 4°C (20 μl of microbeads/10^7 cells). The labeled cell suspension was then passed through the prefilter column in the MACS system placed in the neck of the MACS-VS column. The labeled cells were allowed to react with the magnetized colloid for 10 min, which caused the labeled cells to be retained magnetically while the nonlabeled cells passed through the column. The column was then washed three times with three column volumes of MACS buffer per wash, after which the MACS-VS column containing the reacted cells was removed from the MACS magnetic collar; the retained cells were eluted, and the labeled cells were collected. This positive fraction was then reacted with another fresh MACS-VS column, and the procedure was repeated. At each step, cell fractions were collected and either counted or plated. In addition, samples were collected before and after immunomagnetic enrichment/purging and assayed by FACS to determine recovery and purity.

**“The Combined Purging Techniques”: MACS followed by ad-v Purging.** To create a clinically useful purging technique that could eliminate 5–7 logs of contaminating BCCs, we used the MACS column to enrich the putative CD34+ stem cells, and then followed this with our ad-v purging to remove any residual epithelium.

To test this system, we created an artificial system in which PB-MNCs from healthy donors were added with an artificial burden of contaminating BCCs (the donors were given granulocyte-CSF 5 days earlier to mobilize the CD34+ cells). Before the addition of BCCs, the PB-MNC collections were lethally irradiated (40 Gy) to abrogate colony formation. The initial number of PB-MNCs ranged from 5 × 10^5 to 7.2 × 10^5 and were added to 4 × 10^6 BT-474 (purge 1), 2 × 10^6 BT-474 (purge 2), 6.4 × 10^6 MCF-7 (purge 3), and 5 × 10^6 MCF-7 (purge 4).

As described earlier in the section on MACS, the PB-MNC graft plus BCCs was first reacted with the MACS anti-CD34 immunomagnetic colloid and then passed over the magnetic column. The column was washed three times to remove most non CD34+ cells, including the BCCs, and then removed from the magnetic ring. The CD34+ enriched cells were collected and again placed over a fresh MACS column, and the procedure was repeated. At this point, an aliquot of cells was removed, and the remaining BCCs, which are substantially larger than the HSCs, were counted. FACS was also performed to determine the degree of recovery and purity of the CD34+ population. A total count of cells in the second column was performed, and the mock graft was then treated with the appropriate MOIs of adGAL-TEK for 2 h, after which the cells were plated in Methocult containing 20 μM GCV. Seventy-two h later, the total cell mixture was removed from the Methocult and plated into fresh methylcellulose for a 14-day colony assay.

**RESULTS**

ad-v have been used successfully to transduce numerous epithelium-based targets (12, 28, 29). To selectively eliminate BCCs from BM or PB-SCs using a gene therapy approach, we tested the utility of ad-v to efficiently and preferentially deliver genes to BCCs. For the initial studies, we used the recombinant ad-v adGAL-TEK (16) as both the reporter construct and eventually as the suicide method. We tested adGAL-TEK at an MOI of 50:1 against two BCC lines (BT474 and MCF-7), two fresh patient samples, and MACS-purified HSCs. As shown in Fig. 1, the BCC lines were all efficiently transduced at this low MOI. Specifically, the BCC lines showed results consistent with infection exceeding 96%. The BCC line MCF-7 showed the highest infection, with >98% of the cells expressing the GAL-TEK marker gene. Next was the BCC BT474 line, with >96% of the cells expressing GAL-TEK. Interestingly, the fresh pa-
**Fig. 1** adGAL-TEK transduction into BCCs and MACS purified HSCs. 

A, the BCC lines, BT474 (a) and MCF-7 (b). Two patient samples, RB (c) and WD (d), and MACS purified CD34\(^+\) HSCs (e and f) were exposed to adGAL-TEK at an MOI of 50:1 for 2 h in 100 µl of PBS with 2% FCS, as described in “Materials and Methods.” Forty-eight h after transduction, cells were assayed via FACS for β-gal expression using medium containing C\(_{12}\)-FDG. Control cells were mock infected but reacted with the C\(_{12}\)-FDG-containing medium. The data shown are the percentage of β-gal-positive cells shown by FACS analysis. **Top row:** con, control, which represents the mock-infected cells; **bottom row:** inf, infected cells transduced with adGAL-TEK for 48 h.
tient samples were also efficiently transduced, with each showing >96% expression. In contrast, the MACS purified CD34+ HSCs showed no infection, suggesting that these cells were either poor targets or poorly expressing of the ad-v-delivered genes. Our results suggest that BCCs are better targets than CD34+ cells.

Adenovirus-mediated Reporter Gene Expression in BCCs and Purified HSCs. A major limitation to the use of reporter genes is that the assay methodology is not sensitive enough to detect the low levels of expression or activity. We were also concerned that the results we observed might not be due to the adGAL-TEK vector alone. Because of these concerns, we evaluated the same cell lines and MACS purified HSCs using two other ad-v systems; we also used a more sensitive detection assay system (Promega Corp. package insert). We tested the recombinant adenoviral vectors Av1LacZ4, which encodes for the E. coli LacZ- (β-gal) gene and the ad-v Av1Lu, which encodes for the firefly luciferase gene at an MOI of 50:1 in the BCC lines MCF-7 and BT474, and this was increased to a moderately toxic MOI of 500:1 in MACS purified CD34+ HSCs to reveal any possible effects.

As shown in Fig. 2A, two BCC lines showed high levels, of β-gal activity with MCF-7 cells showing 4342.5 RUs of activity compared with 1.75 RUs in uninfected cells and BT474 cells showing 2468 RUs of activity compared with 1.23 RUs in uninfected cells. Conversely, we observed minuscule amounts of β-gal activity ranging from 1 to 54.5 RUs in the MACS purified CD34+ HSCs with the highest activity occurring in cells infected with an MOI of 500:1. Compared with the response to the MOI of 50:1 used to infect BCCs, HSCs showed minimal β-gal activity.

We observed similar results using the more sensitive luciferase reporter system (Fig. 2B). Specifically, the MCF-7 cell line showed a high level of infection and expression (42,742 RLUs). The BT474 cell line also showed a high level of activity (28,870 RLUs), although somewhat lower than that observed for the MCF-7 cell line. In contrast, the MACS purified HSCs showed no or very low levels of infection and expression. The CD34+ cells infected with an MOI of <500:1 showed an activity of 34.7 RLUs, which was almost equal to background. However, we observed low (780 RLUs) but definitely detectable levels of luciferase activity in the CD34+ cells that were transduced at an MOI of 500:1. Our collective data showed that reporter gene expression levels were two to three orders of magnitude lower in the MACS purified HSCs than in the BCCs.

We also performed RT-PCR analysis of MACS purified CD34+ cells and the MCF-7 cells infected by adGAL-TEK to detect adenovirus specific β-gal expression (Fig. 2C). We detected a positive β-gal amplimer only in the BCC MCF-7 at an MOI of 50:1, suggesting efficient gene transfer. We did not detect any signal in the CD34+ cells at the lower MOIs but did so at 100:1 or more. These RT-PCR results are consistent with the reporter gene expression levels, suggesting that at the low MOIs and short incubation times we used, adGAL-TEK and the other ad-v tested did not efficiently transfer genes to CD34+ HSCs (30). This possible restriction of adenovirus infection to human HSCs supports the use of this vector in a human HSC purging strategy.

AdGAL-TEK Sensitization of BCCs to GCV. To determine whether adGAL-TEK can confer sensitivity to GCV, MCF-7 and BT-474 cells were transduced with adGAL-TEK at an MOI of 50:1 for 1 h. Av1LacZ4 was used as a control to assess the possible toxicity associated with adenoviral gene transfer. Infection of both BCCs with Av1LacZ4 at an MOI of 50:1 had little effect on cell viability (data not shown). More importantly, however, adGAL-TEK infection conferred sensitivity to GCV on all of the BCC lines tested, whereas cells transduced with Av1LacZ4 were insensitive to GCV.

One additional concern we had was that GCV might have toxic effects on the CD34+ HSCs. Therefore, we tested a range of GCV concentrations, which proved to be toxic to the GAL-TEK-transduced cells but not to HSCs. We had also determined that long-term (>4 days) exposure to GCV, even at less than 10 μM, reduced the cloning efficiency of MACS purified HSCs (data not shown). To further evaluate this, we exposed GAL-TEK-transduced cells and CD34+ cells to 20 μM GCV for 72 h. This proved to be fully toxic to the GAL-TEK-transduced cells, but the CD34+ cells where left with full clonogenic capacity. We also assessed the BCC lines (MCF-7 and BT-474) transduced for 2 h with adGAL-TEK. As shown in Fig. 3, the viability of the MCF-7 and BT-474 cells decreased substantially over 7 days, even after the removal of GCV on day 3. Both adGAL-TEK-infected BCC lines tested were <50% viable by day 3. The same BCCs infected with Av1LacZ4 showed no sensitivity to GCV.

Treatment Effects of adGAL-TEK and GCV on Progenitor Cell Clonogenicity. A potential adverse effect of ad-v delivered suicide genes, and the prodrug GCV is nonspecific toxicity to the hematopoietic progenitor cells. We therefore assessed the effects of adenovirus infection and GCV, alone and in combination, on the total CFU capacity (Table 1). Infection with adGAL-TEK alone at an MOI of 50:1 for 2 h had little or no effect (784 ± 138 CFUs), similar to the results we observed with mock-infected cells (805 ± 126 CFUs). The addition of the 20 μM GCV alone also had little or no effect (806 ± 146 CFUs), as did infection of HSCs with adGAL-TEK followed by 20 μM GCV (786 ± 134 versus 805 ± 126 CFUs).

We were also interested in the possibility of augmenting gene transfer by increasing the MOI. We therefore tested higher adenovirus MOIs, ranging from 100:1 to 500:1. At an MOI of 100:1, we noted a 60% decrease in the total CFUs from 805 to 350. We also observed a dramatic decrease in the clonogenic potential of HSCs from 805 to 65 CFUs (a >90% decrease) when the MOI was escalated to 500:1. Because cell kill can usually be increased by increased GCV concentrations, we also tested drug concentrations ranging from 50 to 500 μM. When the GCV concentration was increased to 50–100 μM, we observed a dramatic decrease in total CFUs from 805 to 306 (a 55% decrease), and we observed a massive 98% reduction in colony formation from 805 to 19 because of nonspecific GCV toxicity at a GCV dose of 500 μM.

These results demonstrate that adenovirus infection at an MOI of 50:1 for 2 h with or without 20 μM GCV has little effect on the colony-forming capabilities of the primitive progenitor cells.
Fig. 2 Adenovirus-mediated reporter gene enzyme expression and RT-PCR for adenovirus-specific transcripts in BCCs or MACS purified HSCs. The BCCs MCF-7 and BT474 were exposed to Av1LacZ4 (A) or Av1Lu (B) at an MOI of 50\textsuperscript{1} for 2 h as described in "Materials and Methods." MACS purified HSCs were exposed to various MOIs ranging from 0 to 500\textsuperscript{1} of the same adenovirus vectors for 2 h. Forty-eight h after transduction, the cell lysates were analyzed for reporter gene enzyme activity using the respective assay kits. The results shown represent average reporter gene enzyme activity levels for three infections; bars, SE. C, RT-PCR, incorporating [\textsuperscript{32}P]dCTP, was performed on either MACS purified CD34\textsuperscript{1} cells or the BCC MCF-7 48 h after infection with adGAL-TEK at MOIs ranging from 0:1 to 500:1 for the CD34\textsuperscript{1} cells and at an MOI of 50:1 for the BCC MCF-7. Arrows, the 325-bp-positive \(\beta\)-gal amplimer (top arrow) and the 170-bp amplimer of the \(\beta_2\)-macroglobulin control gene (bottom arrow).

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C

\[\text{Cell Type: CD34}^+ \quad \text{MCF-7}\]
Cytotoxic effects of adGAL-TEK infection and GCV addition on BCC. The BCC lines, MCF-7 and BT474, were exposed to either adGAL-TEK or AvILacZ4 (control) for 2 h at an MOI of 50:1. Cells were then plated in Falcon 96-well plates at a density of 2000 cells/well and exposed to medium containing 20 μM GCV for 72 h, after which fresh non-GCV-containing medium was administered for the remainder of the experiment. On days 0, 2, 4, 6, and 8, cells were assayed to determine viability using CELL TITER 96 AQ. The results shown represent the average of three wells per time point; bars, SE.

Fig. 3  Cytotoxic effects of adGAL-TEK infection and GCV addition on BCC. The BCC lines, MCF-7 and BT474, were exposed to either adGAL-TEK or AvILacZ4 (control) for 2 h at an MOI of 50:1. Cells were then plated in Falcon 96-well plates at a density of 2000 cells/well and exposed to medium containing 20 μM GCV for 72 h, after which fresh non-GCV-containing medium was administered for the remainder of the experiment. On days 0, 2, 4, 6, and 8, cells were assayed to determine viability using CELL TITER 96 AQ. The results shown represent the average of three wells per time point; bars, SE.

Table 1  Treatment effects on progenitor cell colony formation

<table>
<thead>
<tr>
<th>ADENOVIRUSa</th>
<th>GCVb</th>
<th>Colonies</th>
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<tr>
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<td>+100 μM</td>
<td>306 ± 98</td>
</tr>
<tr>
<td>–</td>
<td>+500 μM</td>
<td>19 ± 7</td>
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a Diluted in 1 mM Tris (pH 7.4), 10% glycerol, incubation for 2 h.
b Diluted in PBS, incubation with GCV for 72 h.
c # of colonies (day 14).

d Colonies are expressed as mean ± SE.

Table 2  Cytotoxic killing of BCC by adGAL-TEK purging

<table>
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<tr>
<th>Starting cell number</th>
<th>BT474</th>
<th>HSC (lethally irradiated)</th>
<th>NO treatment</th>
<th>AdGAL-TEK (50:1)</th>
<th>GCV (20 μM)</th>
<th>AdGAL-TEK + GCV</th>
<th>Log cell kill</th>
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<td>2 × 10⁵</td>
<td>1440 ± 250d</td>
<td>1417 ± 178</td>
<td>1380 ± 188</td>
<td>11 ± 15</td>
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<td></td>
<td>2 × 10⁵</td>
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<td>188</td>
<td>188</td>
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a Number of surviving colonies at day 12 (corrected for plating efficiency).

Cytotoxic Reduction of BCCs Cocultured with Progenitor Cell Preparations. The finding that adenovirus preferentially transduces BCCs over MACS purified HSCs suggested that adenovirus-mediated gene transfer into a coculture containing both BCCs and purified HSCs would result in BCCs that were positive for the transgene. If this transgene were the HSVtk gene (or GAL-TEK), then these cells would be sensitive to GCV and die after the addition of GCV. One of our concerns, however, was that the addition of the purified human HSCs into the coculture would reduce or prevent ad-v gene transfer to the BCCs. To test this, we therefore developed a coculture assay in which 10⁷ lethally irradiated 40 Gy HSCs (our mock stem cell graft) were added with various amounts of BT474 cells (the addition of 2 × 10³, 2 × 10⁴, or 2 × 10⁵) to achieve a 0.02 to 2.0% contamination level, respectively. These cocultures were then transduced for 2 h at an MOI of 50:1, using a particle count, which accounted for both the HSCs and the additional BCCs. After the cells were transduced, we plated the cocultures in a 12-day methylcellulose assay to determine the number of clonogenic BCCs remaining after transduction with and without the addition of GCV. As shown in Table 2, at the highest level (2.0%) of contamination, there was a major reduction (4.5–5 log) in the total number of clonogenic cells only when adGAL-TEK transduction was followed by GCV addition. Neither did adGAL-TEK alone have an effect on number of clonogenic cells, nor did GCV by itself have any deleterious effects on colony numbers. At the intermediate level (0.2%) of BCC contamination, we observed a reduction of more than 3 logs of colony formation in adGAL-TEK plus GCV column but observed little or no colony reduction when adGAL-TEK and GCV were administered alone. At the lowest level of contamination (0.02%), we also saw a 3-log reduction in the number of surviving colonies in the adGAL-TEK plus GCV column. Interestingly, we observed a 3–4-log reduction in the number of surviving colonies, regardless of the levels of BCC contamination, suggesting that this eradication strategy is very robust and dependent on the total number of cocultured cells, not just the number of BCCs.

MACS Enrichment and ad-v Purging of Added BCCs in a PB-SC Graft. Clinically, many PB-SC sources are now being immunomagnetically enriched so as to only include more progenitor stem cells in the hopes of achieving a more robust engraftment. A limitation of this immunomagnetic technique, however, is the potential for contaminating BCCs to slip through the enrichment column and end up in the purified graft. We therefore decided that this CD34⁺-enriched progenitor cell graft would be an excellent starting product to test our purging
technique. To do so, we created an artificial system in which PB-MNCs from healthy donors were added with an artificial burden of BCCs, as described in “Materials and Methods.” As shown in Fig. 4B, the MACS enrichment of CD34+ cells resulted in a 1.5–3-log depletion of BCCs, regardless of the initial level of BCCs. However, when CD34+ enrichment was followed by adGAL-TEK infection and the addition of GCV, there was a total 5–7-log reduction in starting number of BCCs. This is shown most dramatically in purge 1 in which 4.4 x 10^4 BCCs remained after CD34 enrichment, but we could detect only 28 colonies (range, 12–35), compared with more than 5000 colonies after adGAL-TEK infection alone. In purge 2, ~4.6 x 10^4 BCCs remained after CD34 enrichment; after adGAL-TEK infection alone, only 13 viable colonies remained after adGAL-TEK plus GCV administration, and 4527 colonies remained after adGAL-TEK infection alone in purge 3, ~4.2 x 10^4 BCCs (only a 1.5-log depletion) remained after CD34 enrichment; after adGAL-TEK infection alone, only 10 colonies (range, 3–17) remained, and 4103 colonies remained after adGAL-TEK infection alone. In purge 4, 4.7 x 10^4 BCCs remained after CD34 enrichment, after adGAL-TEK infection alone, 12 colonies (range, 8–17) remained, and 4873 colonies remained after adGAL-TEK infection alone.

At the beginning of the CD34+ enrichment and after the

Fig. 4 MACS enrichment of CD34+ cells followed by adGAL-TEK infection and GCV addition. We created a artificial system in which PB-MNCs from healthy donors were added with BCCs. This graft first underwent MACS CD34+ enrichment and then adGAL-TEK infection + GCV addition. A, the sequence of events for our two-step enrichment/ purging technique. B, the results shown are those obtained at various steps shown in A. Step 1, the starting numbers of cells added (cell counts); Step 2, the number of purified CD34+ cells (cell counts) and the approximate number of BCCs remaining (cell counts); Step 4, the total colonies counted in the two plates of cells treated either with adGAL-TEK alone or adGAL-TEK + GCV (corrected for plating efficiency in Myelocult).
second MACS column (but before ad-v purging), a cell sample was removed that was used for counting the total number of CD34+ cells and subjected to FACS to determine purity after the enrichment procedure. As shown in Table 3, with each enrichment we routinely recovered >70% of the starting CD34+ cells (we estimate ~15% cell loss/column run) and obtained purities exceeding 98%.

### DISCUSSION

High-dose chemotherapy with the subsequent reinfusion of autologous peripheral blood progenitor cells is increasingly recognized as a safe and efficient treatment for cancer patients. The presence of residual tumor cells in the autograft, however, remains a major problem, and the role these cells play in recurrence is unknown. Recent data from gene marking studies do, however, suggest that the reinfusion of contaminating tumor cells in some patients with malignant disease contributes to relapse [e.g., acute myelogenous leukemia, neuroblastoma (31), and chronic myelogenous leukemia (32)]. In particular, Sharp et al. (33) reported that women with breast cancer who receive breast cancer culture-negative BM remained disease free longer than did women who received culture-positive BM. In addition, a retrospective study conducted by Fields et al. (34) suggested that patients who received cytokeratin K-19-negative BM, as shown by RT-PCR, had a longer disease-free time to relapse than did patients who received K-19-positive BM. Taken together, the results of these studies suggest that autografts that are effectively purged of contaminating BCCs are associated with a longer disease-free survival. Presently at The University of Texas M. D. Anderson Cancer Center, women who have detectable breast cancer contamination in the BM are not eligible for autologous BM transplantation and must undergo allogeneic transplantation, which is associated with an increased likelihood of graft rejection and graft-versus-host disease. Because of these problems, we have devised a two-step enrichment/purging technique that can be used in a clinical setting to eradicate up to 7 logs of contaminating BCCs in the graft. The main component of the strategy is the ad-v purging method, which relies on the preferential infection of contaminating BCCs over HSCs. The immunomagnetic enrichment of the CD34+ HSCs depletes CD34+ cells (or contaminating BCCs), as well as increases the number of progenitor cells in the graft, which theoretically increases the likelihood of engraftment. This initial selection of CD34+ cells thereby substantially reduces the number of CD34+ cells (2–3 logs) to be purged by ad-v, and thus much less ad-v has to be applied, hopefully reducing any ad-v-associated toxicity.

A concern we had was that BCCs from metastatic lesions would not be susceptible to ad-v transduction. There are reports that suggest that primary tumors are not infectable with ad-v; however, our data suggest that these metastatic lesions were highly infectable by ad-v, and a recent report suggests that metastatic tumor may up-regulate αV integrins as a requirement for metastasis (35). We feel that our data support this literature, and that this purging technique may work better than we expect due to this up-regulation of these primary ad-v receptor molecules.

One major limitation of this purging system is the potential transduction of adenovirus into HSCs. However, we demonstrated that this occurs to a much less extent in HSCs than in BCCs. The reason for this could be that adenovirus infection is a two-step process involving the attachment of the adenoviral fiber protein to the recently described coxsackie-adenovirus receptor (36) and then internalization of the virus through interaction of the adenoviral penton base with integrins αVβ5 and αVβ3. Chen et al. (23) recently analyzed a series of human HSCs and BCC lines and noted that HSCs do not express these integrins, but BCCs do. In addition, Seth et al. (21), who used radiolabeled ad-v and performed Scatchard plot analysis, observed that low-density BM cells have very low levels of ad-v receptors in comparison with BCCs. These collective results indicate that the low level of adenovirus-mediated transduction into CD34+ cells may stem from their lack of the integrins required for internalization. In contrast, three recent studies (37–39) suggest that HSCs can support ad-v transfer with high efficiency. However, a common theme of these studies is that for ad-v to efficiently transduce into HSCs, there must be: (a) long-term incubation (>24 h); (b) relatively high MOIs >100:1; and (c) cytokines in the culture medium. The short-term, low MOI infections we used in our purging strategy should allow for efficient gene transfer into BCCs but not HSCs. If there were ad-v transduction into HSC, this would be minimal, based on the fact that the cytomegalovirus promoter used in our viral constructs was expressed at very low levels (30). Our RT-PCR data further suggest that the HSCs were not efficiently transduced at the low MOIs used. The differential sensitivity of HSCs and BCCs to ad-v infection supports the use of ad-v purging after MACS CD34+ enrichment to eradicate contaminating epithelial cells. Other techniques for “purging” BM preparations have been reported to produce 2–5-log depletion but are plagued by problems. For example, the physical removal of tumor antigen-expressing BCCs via a panel of antibodies and immunomagnetic separations on a magnetic column (26) was reported to produce 5-log reduction but requires the whole population of BCCs to express the correct antigens. Purging with 4-hydroperoxycyclophosphamide can result in a 3–4-log depletion (5), but this also resulted in the marked reduction of colony formation. Other approaches involving the use of microspheres (6) and immunotoxins (7) have also been reported to be associated with varying degrees of success and limited toxicity to the HSCs.

Although MACS enrichment appears to physically separate contaminating BCCs from the HSCs in the graft product, it does so at a low efficiency. Our best result was a 3-log removal. However, these purified cells provide an excellent starting ma-
terial for the ad-v-based purging method. The ad-v (AdGAL-TEK, Av1LacZ4, and Av1Lu) infected the BCCs tested as well as two fresh patient samples with high efficiency. AdGAL-TEK-infected cells were also efficiently killed after a short pulse of 20 μM GCV. In addition, the ad-v purge did not cause any deleterious effects on CD34+ colony formation. We were particularly pleased by the robustness of this ad-v purge in our mixed culture experiment (see Fig. 4B). This was shown in purge 3, in which there was only a 1.5-log depletion of BCCs after MACS but there were only a small number of BCC colonies surviving after adGAL-TEK infection and the addition of GCV. The number was almost equivalent to the number of BCCs surviving from purges 1 and 4 (2–3-log depletion of BCCs). It also does not appear from our experimental data that the effectiveness of the ad-v purge depends on the initial number of cancer cells remaining after CD34+ enrichment.

There are reports suggesting that ad-v purging is an effective technique; however, none of them have been designed to have direct clinical application. In a report of Chen et al. (23), the authors describe an adenoviral construct containing a tumor-specific promoter, MUC1, that drives expression of the HSVtk gene. The data presented suggest that his purging technique is highly specific for the BCC lines MCF-7 and ZF-75–1. In particular, these authors reported an up to 7-log eradication of reporter gene activity. They also observed that limiting dilution assays of ad-v-transduced BCCs resulted in a 5-log depletion of BCCs. Wroblewski et al. (24) also demonstrated that ad-v purging could be effective by showing that ad-v carrying either HSVtk or p53 genes preferentially targets MCF-7 cells in BM. Interestingly, they observed that ad-v-mediated HSVtk expression, followed by the administration of GCV, was not potent enough to eradicate the contaminating MCF-7 cells and suggested the use of adp53 as a more potent suicide gene. In an additional study, Kim et al. (40) used ad-v purging to eliminate prostate carcinoma cells from human BM. These collective findings suggest that, because of the natural ability of ad-v to preferentially infect cells of epithelial origin over those of hematopoietic origin make, ad-v purging is a viable alternative (41). Other possible suicide genes have been considered for these purging strategies, such as the use of bacterial cytosine deaminase and the produrg 5-fluorocytosine, have also been tested, with very similar purging efficiencies (42). One concern with our purging technique since it uses the HSVtk gene/GCV is that only replicating cells will be intoxicated upon GCV administration. Although this may be true, we have detected expression of the adGAL-TEK gene for up to 15 days, and during this time window, the BCCs should become susceptible to GCV killing. We feel that the BCCs that are contaminating the PB-SCs will fall into three categories: (a) terminally differentiated and will therefore not divide and not contribute to relapse; (b) quiescent in culture due to lack of proper growth stimuli and may return to cycle upon proper growth factor signals in vivo; and (c) cycling ex vivo. All three categories of these cells will be infectable with the ad-v and upon reactivation into cell cycle quickly eradicated upon GCV administration.

The use of ad-v for in vivo applications has raised many concerns due to the immunogenicity of the adenovirus; this immunogenicity problem has not been formally tested in an ex vivo transduction setting. It would be expected that the transduced cells could be potentially immunogenic, and that any of the CD34 cells that were nonspecifically infected could also illicit an immune response. However, it is our feeling that because we are giving such a low MOI and using a short incubation period, that very few CD34 cells would be infected, and even if eradicated (due to immunogenicity), would not be an issue in a clinical setting. Our purging strategy is to reinfuse enriched CD34 cells (>95% positive), and the loss of 2–5% of these cells would not have an impact clinically.

The specific finding that adenovirus preferentially infects BCCs over HSCs suggests that ad-v purging is feasible in women with breast cancer. We therefore suggest that, if this enrichment/purging technique is introduced clinically, women undergoing autologous BM transplantation who have detectable BCC contamination have routine PB-SC collection followed by CD34+ enrichment using the MACS, a technique that is still undergoing clinical trials. After this, a quick cell count should be performed and the graft infected with an ad-v carrying a suicide gene (i.e., ad-GAL-TEK or adp53), followed by direct reinfection of the purged graft and GCV administration. On the basis of our data, it is possible to achieve an up to a 5–7-log eradication of contaminating BCCs while enriching the CD34+ cells so they constitute >98% of the total graft. This technique has the potential to allow all patients, regardless of breast cancer contamination levels, to be eligible for autologous transplantation by ensuring the removal of all BCCs from the graft. We conclude that this enrichment/purging technique can also be used safely and effectively to remove contaminating epithelium, such as prostate and ovarian cancer cells, in other mixed hematopoietic grafts.

ACKNOWLEDGMENTS

We thank Ling Chen for helpful discussions and the Departments of Blood and Marrow Transplant and Surgical Oncology for the apheresis products and patient samples.

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Purging of Contaminating Breast Cancer Cells from Hematopoietic Stem Cell Grafts by Adenoviral GAL-TEK Gene Therapy and Magnetic Antibody Cell Separation

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